Reduction of Epidermal Abnormalities and Inflammatory Changes in Psoriatic Plaques During Treatment With Vitamin D_3 Analogs

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Vitamin D_3 analogs interfere with various aspects of epidermal growth, inflammation, and cellular differentiation. Most data are derived from *in vitro* studies. In the present review, the *in vivo* effects of vitamin D_3 analogues on the psoriatic plaque are discussed. Calcipotriol, tacalcitol, and calcitriol in ointment modulate aspects of epidermal growth, differentiation, and inflammation. Immunohistochemical studies suggest that the inflammatory changes might be more expressed after treatment with calcitriol and tacalcitol. Flow cytometric quantification of the percentage of cells in SG_2M phase and of keratin 10–positive cells revealed that calcipotriol reduced both indices significantly during treatment of psoriatic plaques. Flow cytometric analysis of epidermal cell suspensions using triple labeling for epidermal proliferation, expression of keratin 10, and vimentin permits a quantitative assessment of DNA synthesis selectively in the basal cells of the epidermis, an estimation of the distribution of the basal and suprabasal compartments, and a quantification of the distribution of mesenchymal and nonmesenchymal cells. Using this approach, the interference of tacalcitol with growth control of basal cells was demonstrated. Remarkably, recompartmentalization of basal and suprabasal cells and mesenchymal and nonmesenchymal cells proved to be inconspicuous during this treatment. *Key words: calcitriol/calcipotriol/tacalcitol. Journal of Investigative Dermatology Symposium Proceedings 1:78–81, 1996*

In the last decade, vitamin D_3 analogs have become an important approach in the treatment of psoriasis. Calcipotriol is available as a routine treatment in many countries (Binderup and Kragballe, 1992). In Japan, tacalcitol (1α, 24-dihydroxyvitamin D_3) is a first line of treatment for psoriasis (Nishinura *et al*, 1993). Calcitriol (1α,25-dihydroxyvitamin D_3) is the naturally occurring, active vitamin D_3 and has been investigated with respect to its antipsoriatic efficacy and safety (Langner *et al*, 1992).

The aim of the present review is to evaluate the actions of calcipotriol, tacalcitol, and calcitriol on psoriatic skin during topical treatment.

VITAMIN D_3 ANALOGS INHIBIT EPIDERMAL PROLIFERATION, ENHANCE NORMAL KERATINIZATION, AND MODULATE INFLAMMATION

Calcipotriol, calcitriol, and tacalcitol bind to the vitamin D receptor (VDR), and the complex binds to vitamin D response elements within promoter regions of vitamin D response genes (Ozone *et al*, 1991). VDR belongs to the steroid receptor superfamily together with the receptors for retinoids, estrogen, thyroxine, and glucocorticosteroids. Evidence is accumulating that the receptors within this family do not act in isolation, but interact. As such, the heterodimer formation of VDR and the RXR-α receptor is important because the heterodimer exerts a more potent binding with vitamin D response elements compared with VDR alone (Kliewer *et al*, 1992).

In addition to these nuclear mechanisms, vitamin D_3 analogs have a direct effect on calcium entry. Indeed at physiologic concentrations, calcitriol has been shown to enhance calcium entry into keratinocytes (Brittener *et al*, 1991).

Either via nuclear mechanisms or by increased entry of calcium into the cell, vitamin D_3 enhances the production of inositol trisphosphate and 1,2-diacylglycerol (MacLaughlin *et al*, 1990). Another important effect of vitamin D_3 analogs in cell signaling is the translocation of protein kinase C from the cytosolic to the membrane position (Yada *et al*, 1989).

At the cellular level, vitamin D_3 analogs have been shown to inhibit proliferation of keratinocytes and to enhance cornified envelope formation (Binderup and Bramm, 1988; Kragballe and Wildfang, 1990). At the molecular level, vitamin D_3 analogs enhance transglutaminase activity, which is a crucial enzyme for cornified envelope formation (Hosomi *et al*, 1983; Lee *et al*, 1989; Matsunaga *et al*, 1990). In contrast to retinoids, vitamin D_3 analogs do not modulate the transcription of keratin genes. These compounds specifically interfere with the last step of differentiation (Regnier and Darmon, 1991).

Vitamin D_3 analogs have diverse effects on inflammation control. Interleukin (IL)-1–induced T-lymphocyte proliferation is inhibited by active vitamin D_3 (Tsoukas *et al*, 1984). Production of IL-2 and IL-6 by T lymphocytes and accumulation of mRNA for IL-2, interferon-gamma, and granulocyte-macrophage colony-stimulating factor increase in the T lymphocyte after incubation with calcitriol (Tsoukas *et al*, 1984; Gupta *et al*, 1989; Hustmyer *et al*, 1991; Lemire, 1992). Macrophages are activated by calcitriol, and

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Abbreviations: TP_3, TO-PRO-3 iodide; VDR, vitamin D receptor.

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Table I. Monoclonal Antibodies Used for the Immunohistochemical Assessments[a]

Cell Biologic Feature	Antibody
Recruitment of cycling cells	Ki-67
Keratin 16 expression	K_s 8.12
Involucrin	Mon-150
Filaggrin	Anti-filaggrin (BT 576)
Transglutaminase I	Anti-TG-ase I (IgG_{2a})
Pan T cells	T 11
Polymorphonuclear leukocytes	Anti-elastase
Monocytes (CD14)	WT 14
Langerhans cells	OKT 6

[a] The immunohistochemical procedures have been described before (de Jong and van de Kerkhof, 1991; Gerritsen *et al*, 1993, 1994, 1995).

the interferon-gamma–induced expression of human leukocyte antigen–D on keratinocytes is inhibited by calcitriol (Lehman and Gray, 1984; Petrini *et al*, 1991; Tone *et al*, 1991; Oberg *et al*, 1993). Archidonic acid release and migration of polymorphonuclear leukocytes (PMN) are inhibited by vitamin D_3 analogs (Tanaka *et al*, 1988).

VITAMIN D_3 ANALOGS ARE CLINICALLY EFFECTIVE IN CHRONIC PLAQUE PSORIASIS, PUSTULAR PSORIASIS, AND ICHTHYOSES

Based on the *in vitro* observations, it is feasible that calcipotriol, tacalcitol, and calcitriol are effective in skin disorders characterized by epidermal hyperproliferation, abnormal keratinization, and inflammation. Indeed, the therapeutic efficacy of active vitamin D_3 is not restricted to chronic plaque psoriasis, but also affects pustular psoriasis (Berth Jones *et al*, 1992). Active vitamin D_3 has proved effective in some disorders of keratinization (Kragballe *et al*, 1995).

REDUCTION OF IMMUNOHISTOCHEMICAL CHANGES DURING TREATMENT

Three clinical studies were carried out in patients with chronic plaque psoriasis to assess the effect of twice-daily applications of calcipotriol (50 μg/g) in ointment, once-daily applications of tacalcitol (4 μg/g) in ointment, and twice-daily applications of calcitriol (3 μg/g) in ointment (de Jong *et al*, 1991; Gerritsen *et al*, 1993, 1994, 1995). In previous dose-response studies, these concentrations proved to provide optimal clinical efficacy. In total, eight patients were included for treatment with calcipotriol, ten patients for treatment with calcitriol, and ten patients for treatment with tacalcitol in ointment. Before treatment and at various times, biopsy specimens were taken and processed for immunohistochemical assessment.

The immunohistochemical methods have been described before (de Jong *et al*, 1991; Gerritsen *et al*, 1993). In brief, samples were snap-frozen in liquid nitrogen, and consecutive sections were cut. Epidermal growth, differentiation, and inflammation characteristics were analyzed using an indirect peroxidase technique. **Table I** summarizes the monoclonal antibodies used in the studies.

Although the concentrations of active vitamin D_3 analogs used in the treatments were different, the clinical improvement was comparable. Approximately a 50% reduction of the psoriasis area severity index was achieved for all three analogs (Gerritsen *et al*, 1994, 1995).

The maximum observation period for calcipotriol ointment was 12 wk (de Jong *et al*, 1991). After 2 wk of treatment with calcipotriol ointment, a significant decrease in the number of cycling epidermal cells (Ki-67-positive nuclei) was observed. During the subsequent observation period, the number of Ki-67-positive nuclei showed only a modest reduction beyond that reached after 2 wk of treatment. Keratin 16 (K_s 8.12 binding of the suprabasal compartment of the epidermis) tended to decrease between 4 and 8 wk of treatment. After 1 wk of treatment, only small microabscesses of PMN were observed, and after 4 wk of treatment, no microabscesses were observed at all. The accumulation of T cells showed only a small reduction in the 12-wk treatment period, reaching borderline significance after 4 wk. The accumulation of monocytes and macrophages did not show a significant modulation during calcipotriol treatment.

The maximum observation period for calcitriol treatment was 4 wk (Gerritsen *et al*, 1993). During this treatment period, the maximum reduction of the number of Ki-67-positive epidermal nuclei was observed after 4 wk of treatment. In this study, the numbers of involucrin-positive and transglutaminase-I-positive cells decreased, and the number of filaggrin-positive cells increased. Pan T cells and PMN decreased markedly during the observation period.

The maximum observation period for tacalcitol treatment was 8 wk (Gerritsen *et al*, 1994, 1995). During this interval, the numbers of Ki-67–positive nuclei and the involucrin-, keratin-16-, and transglutaminase-positive cell layers had diminished substantially, whereas the number of filaggrin-positive cell layers had increased. Tacalcitol had a remarkable effect on the number of PMN, T cells, and monocytes in the dermis and epidermis of the psoriatic lesion. In contrast, the ointment without tacalcitol had no significant effect on these markers, apart from a reduction in the number of Ki-67-positive nuclei.

These immunohistochemical studies suggest that the three vitamin D_3 analogs have an effect on epidermal growth, keratinization, and cutaneous inflammation **(Table II)**. The reduction of the inflammatory infiltrate was most pronounced after treatment with tacalcitol. Definitive conclusions on the relative potencies of these analogs require comparative studies.

In various centers, the *in vivo* effects of vitamin D_3 analogs have been studied. On the one hand, these studies suggest a reduction of keratin-16- and keratin–17-positive cells and normalization of keratin-5- and keratin-10-positive cells, with only modest changes of the immune system (Verburgh *et al*, 1989; Holland *et al*, 1990; Berth Jones *et al*, 1991; de Jong *et al*, 1991). One study suggested, however, that calcipotriol treatment causes a reduction of helper T cells with a relative persistance of epidermal phenomena (Malet *et al*, 1990). In another study, it was shown that calcipotriol treatment reduced IL-6 staining without altering tumor necrosis factor-α staining (Oxholm *et al*, 1989). Light and electron microscopic investigations showed that tacalcitol treatment induced a normal keratin pattern and the reappearance of normal keratohyalin granules (Ueda *et al*, 1989).

The general pattern of the immunohistochemical response is a pronounced reduction of epidermal proliferation and the accumulation of PMN. Other topical treatments such as corticosteroids (de Jong *et al*, 1995) and dithranol (de Jong and van de Kerkhof, 1992) were shown to have a similar *in vivo* response: the reduction of epidermal proliferation and the accumulation of PMN as a nonspecific effect. The reduction of PMN, T cells, and monocytes,

Table II. Reduction of Epidermal Proliferation and Modulation of Keratinization and Inflammation During Treatment With Vitamin D_3 Analogs[a]

	Calcipotriol (50 μg/g Twice Daily)	Calcitriol (3 μg/g Twice Daily)	Tacalcitol (4 μg/g Once Daily)
Ki-67	⇓⇓	⇓⇓	⇓⇓
Keratin 16	↓	ND	↓
Involucrin	ND	↓	↓
Filaggrin	ND	↑	↑
Transglutaminase	ND	↓	↓
Polymorphonuclear leukocytes	⇓⇓	⇓⇓	⇓⇓
Pan T cells	=/↓	↓	↓
Monocytes	=	ND	↓
Langerhans cells	=	ND	=

[a] Quantification of the responses has been described before (de Jong and van de Kerkhof, 1991; Gerritsen *et al*, 1993, 1994, 1995). Abbreviations: =, no modulation; ↓, slight decrease after treatment; ⇓⇓, marked decrease after treatment; ↑, slight increase after treatment; ND, not done.

however, was relatively more expressed during treatment with calcitriol and tacalcitol than with calcipotriol.

The antiproliferative action and the inhibition of PMN functioning are observed both *in vitro* and *in vivo*. In contrast to the activation of monocytes *in vitro*, tacalcitol induced a reduction of the accumulation of monocytes in the psoriatic plaques. And in contrast to the substantial inhibition of T-cell functioning *in vitro*, these cells tended to persist during treatment with vitamin D_3 analogs. In contrast to enhancement of transglutaminase I activity *in vitro*, during treatment with vitamin D_3 analogs the number of transglutaminase-I–positive cell layers decreased.

To elucidate further the *in vivo* action profile of vitamin D_3 analogues, quantitative comparative studies are required.

FLOW CYTOMETRIC QUANTIFICATION OF EPIDERMAL CHANGES DURING TREATMENT

To quantify epidermal hyperproliferation in the psoriatic plaque before and during treatment with calcipotriol and betamethasone, flow cytometric assessment was carried out of DNA distribution and keratin 16 expression (de Mare *et al*, 1990). In this study, a group of 20 patients with chronic plaque psoriasis were treated twice daily with calcipotriol (50 μg/g) in ointment and betamethasone in ointment in a double-blind trial with a left-right comparison. Before treatment and after a treatment period of 6 wk, razor-blade biopsy specimens (0.5 mm thick, 4 mm in diameter) were taken from two lesions at both sides. Epidermal cell suspensions were prepared using a trypsinization procedure, as described previously (Bauer *et al*, 1980). To assess keratin 16 expression, we incubated the cell suspensions with the monoclonal antibody K_s 8.12 and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG as second antibody (de Mare *et al*, 1990). Propidium iodide was added to measure relative DNA content per cell (de Mare *et al*, 1990). Five thousand cells of each sample were analyzed using an Ortho 50H flow cytometer equipped with a 5-W argon laser tuned at 488 nm. Both treatments induced similar reductions of these indices. The values, however, were still well above the normal ranges (percentage K_s 8.12 cells: 1.8 ± 1.0 [mean ± SEM] [van Erp *et al*, 1989] and percentage cells in SG_2M phase: 4.0 ± 0.7 [Bauer *et al*, 1981]).

Although this approach permits quantification of proliferation characteristics in epidermal cells, the methodology is complicated by the fact that suprabasal cells and mesenchymal cells are not excluded from the assessment.

Recently, a new methodology was developed using a triple labeling approach. Per-cell DNA content was measured using the new DNA stain TO-PRO-3 iodide (TP3); anti-vimentin was used to identify all nonkeratinocytes (infiltrate cells and other mesenchymal cells); RKSE 60 staining was carried out to assess keratin 10 expression, which indicates whether a cell belongs to the suprabasal cell population. Cell suspensions were prepared as described before, and the staining procedures have been described previously (Bauer and Boezeman, 1983; van Hooijdonk *et al*, 1995). The second step of the indirect immunofluorescent staining was performed with monoclonal goat antibodies against mouse IgG_1 and IgG_{2a}, conjugated to phycoerythrin and fluorescein-isothiocyanate (FITC), to assess RKSE 60 binding and anti-vimentin binding, respectively. Phycoerythrin and FITC were excited with an air-cooled argon laser (633 nm), and TP_3 was excited with an HeNe laser (488 nm). After electron compensation for spectral overlap, which was minimal in the case of TP_3, fluorescence was measured using band-pass filters at 525 nm (green FITC), 575 nm (orange, phycoerythrin), and 675 nm (red, TP_3). The ratio of area to peak of the red signal was used to discriminate between doublets of diploid cells and real tetraploid cells (Bauer and Boezeman, 1983). After setting the appropriate gates with the Elite software percentages and with the aid of Multicycle software, the percentages of vimentin- and keratin-10–positive cells and the percentage of cells in SG_2M phase were calculated. From these data, the percentage of cells in SG_2M phase within the pure basal cell population was calculated.

In total, 20 patients participated in a left-right comparative study between tacalcitol (4 μg/g) in ointment and the ointment base only (Glade *et al*, 1995). Applications were done once daily. Razor-blade biopsy specimens were taken before treatment and after 8 wk of treatment from two lesions at both sides. Cell suspensions were prepared, and triple-label flow cytometric assessment was carried out according to the methods described above. The sides treated with tacalcitol showed a mean reduction of the psoriasis area severity score of 48%. The psoriasis area severity score of the lesions treated with placebo ointment was decreased by 28%.

In the psoriatic lesions before tacalcitol treatment, the percentage of basal cells in SG_2M phase was 20.0 ± 1.9% (mean ± SEM); after 8 wk of treatment, the percentage was reduced to 13.2 ± 1.1%. This reduction was statistically significant ($p \leq 0.01$). At the placebo-treated sides, the pretreatment value was 17.8 ± 1.8%, and the value after 8 wk of placebo treatment was 15.1 ± 1.2%.

The pronounced reduction of this cell-cycle kinetic marker during tacalcitol treatment illustrates the potent effect of tacalcitol on epidermal hyperproliferation *in vivo*. Triple-label flow cytometry has proved to be an adequate tool to restrict the analysis of indices for epidermal proliferation to the basal cells, even within a population as heterogeneous and complex as the inflamed epidermis of the psoriatic lesion.

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